Residue Levels in Honey after Colony Treatment with the Antibiotic Tylosin

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Abstract

Residue levels in honey were determined using a microbiological assay after the antibiotic tylosin was applied to honey bee colonies. The antibiotic was applied as a dust (200 mg or 1000 mg in 20 g confectioners sugar) three times, one week apart, and both brood and surplus honey were sampled and analyzed during and following the treatment period. Tylosin concentrations declined over time in all samples from all colonies. In surplus honey from colonies treated with a total of 600 mg, tylosin concentrations declined from an average of 1.31 ppm in honey sampled during the treatment period to 0.16 ppm three weeks after the last treatment. Based on US per capita consumption, exposure to tylosin from honey is less than that from other agricultural products.

INTRODUCTION

American foulbrood disease (AFB) is a highly contagious disease of young honey bee brood caused by the bacterium *Paenibacillus larvae* subsp. *larvae* that has been controlled, in part, by the antibiotic oxytetracycline (OTC; = Terramycin®). Reports of foulbrood bacterial resistance to OTC has been documented in the literature (Alippi, 2000; Miyagi *et al.*, 2000), prompting laboratory screening for suitable alternative antibiotics (Kochansky *et al.*, 2001). One of the candidate compounds identified during this screening was the macrolide antibiotic tylosin. This antibiotic has previously been shown to be effective in controlling AFB (Hitchcock *et al.*, 1970; Moffett *et al.*, 1970; Peng *et al.*, 1996; Allippi *et al.*, 1999; Elzen *et al.*, 2002), though no concerted effort was made to achieve registration of this compound for use in bee colonies. In order to gain US Food & Drug Administration (FDA) approval for the use of tylosin in honey bee colonies to control AFB, we undertook a study to determine what residue levels were present in honey when colonies were treated with tylosin under field conditions. To mimic a worst-case scenario under conditions where the largest quantities of residue would be detected, antibiotic treatments were applied during the nectar flow, and honey was sampled during and immediately after treatment, with no withdrawal period.

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MATERIALS and METHODS

Antibiotics and formulations

Tylan® soluble (Elanco Animal Health, Indianapolis IN) was purchased commercially and served as the basis for formulations of tylosin. Tylosin was formulated with powdered sugar (Domino Confectioner's 10X) by mixing in a stainless steel jar mill with lifter bars (U.S. Stoneware, East Palestine OH) for 10 minutes to provide two concentrations: (1) 200 mg tylosin in 20 g sugar (= "1xT") and 1000 mg tylosin in 20 g sugar (= "5xT"). Doses (20 g) of the antibiotic/sugar mixture were weighed into individual 50-ml plastic centrifuge tubes to facilitate application in the field.

Colonies and antibiotic treatment regime

Twelve colonies obtained from McCoy's Sunny South Apiaries (Loxahachie, FL) were established at the ARS US Horticultural Research Laboratory in Ft. Pierce FL on February 22, 2001. All colonies were queenright and contained no surplus honey. Colonies were randomly assigned treatment groups designated 1xT, 5xT (see above) or untreated. Treatments of the antibiotic/sugar mixtures were applied across the top frames in the brood chamber every seven days until a total of three treatments were applied. Four colonies were used for each treatment group.

Colony management and honey collection

Inspection of the brood box prior to the second treatment revealed a nectar flow. Therefore, concurrent with the second treatment, a queen excluder and an empty, shallow honey super were placed on all colonies. On the third treatment date, these supers were removed (and reserved) and replaced with new empty supers to ensure that the honey collected in subsequent weeks would be produced after the third treatment. One week after the third treatment, a frame of honey was removed from each super and replaced with an empty frame. All other frames containing honey were marked with an indelible pen. As the nectar flow was strong, all colonies received an additional honey super. A frame of honey from the brood chamber of each colony was also removed at this time (one week after the third treatment) and replaced with an empty frame. Frames of surplus and brood honey were subsequently removed from each colony weekly, for two additional weeks. This regime allowed for the collection of a total of four surplus honey samples (one sample produced between the second and third treatment and three samples, one-, two-, and three-weeks after the third treatment), and three brood honey samples (one-, two-, and three-weeks after the third treatment). No frames of honey were produced by the fourth week after the third treatment due to the cessation of nectar flow.

Frames containing surplus and brood honey were taken to a field laboratory within 1-hour of removal from the colony, and approximately 20-25g of honey was removed from each frame and placed in individually labeled, 4-oz pre-cleaned glass jars with teflon-lined caps (Scientific Specialties, Randallstown MD) for

transport to Beltsville MD. In Beltsville, all samples were held overnight at - 20 ^{0}C until analyses the next day.

Preparation of Standard Curve

For the preparation of the standard curve, tylosin tartrate was purchased from Sigma (St. Louis, MO). A stock solution was prepared in water at a concentration of 100 mg/L. Honey was collected from a colony in our apiary in Beltsville, MD that had never been treated with antibiotics. Aliquots of honey (5 g) were spiked with the appropriate amount of stock solution to yield 24 antibiotic concentrations ranging from 0 - 40 ppm. These spiked honey samples were diluted with water (10 ml) and loaded on pre-conditioned (1-ml methanol followed by 1-ml water) solid-phase extraction cartridges (Oasis HLB; Waters, Inc., Medford MA). After passage of 1-ml 5% methanol, tylosin was eluted with 1-ml 100% methanol. A subsequent strip fraction of 1-ml acetonitrile did not elute any additional antibiotic. Methanol fractions containing antibiotic were dried *in vacuo* using a Speedvac (Thermo Savant; Holbrook NY), reconstituted in 100 ml aqueous methanol (50%), and a 20 ml aliquot spotted on a paper disk and assayed using the microbiological assay described below. Three replicates were assayed for each concentration.

Honey Analyses

Aliquots (5g) of surplus and brood honey collected in the Ft. Pierce study from treated and untreated colonies were processed within 24h of collection, using the identical extraction protocol and microbiological assay described herein.

Microbiological Assay

A disk diffusion assay as reported by Feldlaufer *et al.* (1993) and Shimanuki and Knox (2000) was used to generate a standard curve and determine residue levels in honey. Known amounts of tylosin (for the standard curve) or honey extracts (for the residue study) were tested against an oxytetracycline-resistant strain of *Paenibacillus larvae* obtained from Minnesota. A stock spore suspension (approximately 2 x 10^{8} spores/mL) was prepared by mixing 3–5 scales (the dried remains of diseased honey bee larvae containing the bacterial spores) with sterile water (9 mL) in a screw-capped tube. Before each use, the suspension was heat-shocked at 80° C for 10 minutes to kill any non-sporeforming bacteria. For the bioassay, 0.2 mL of the stock suspension was spread over the surface of freshly-prepared brain-heart infusion agar (BHI) plates (Difco Laboratories, Detroit, MI), fortified with thiamine hydrochloride (0.1 mg/L), 2% agar, and adjusted to pH 6.6 with hydrochloric acid. Paper disks (No. 740-E; Schleicher and Schuell) treated with either known amounts of tylosin or honey extracts were positioned in the center of the BHI plates and the plates were incubated at 34 °C in the dark. The diameters of the zones of inhibition were measured after 72 hours.

Statistical analyses

For the standard curve, zones of inhibition (n = 3 replicates) were measured and recorded (Table I) for 24 tylosin concentrations ranging from 0 – 40 ppm. We transformed the scale of the antibiotic concentrations by adding 0.1 to all concentrations and then taking the $\log_{10}$ of the results. We regressed the inhibition zone diameters on the transformed antibiotic concentrations as a quadratic polynomial to generate the standard curve shown in Fig. 1. Based on an inverse regression of this standard curve, we could estimate antibiotic concentration in honey collected in our study from measured zones of inhibition. Upper and lower 95% confidence limits on these estimates were calculated following the methods for fiducial limits in Draper and Smith (1981). Based on this model, we could determine with 95% certainty that tylosin was present in samples yielding a zone of inhibition of 15 mm, which corresponds to an estimated level of detection of 0.07 ppm.

RESULTS

A total of 600 mg (for the 1xT dose) and 3000 mg (for the 5xT dose) of tylosin in confectioner's sugar were applied to honey bee colonies over a two week period. Four surplus honey samples (one sample produced between the second and third treatment and three samples one-, two-, and three-weeks after the third treatment), and three brood honey samples (one-, two-, and three-weeks after the third treatment) were collected from these colonies and from untreated colonies and subsequently analyzed. Estimates of mean tylosin concentration in honey for all treatment groups and collection dates are shown in Table II. Tylosin concentrations declined

Table 1. Zones of inhibition resulting from known concentrations of tylosin tartrate used to generate the standard curve [1].

concentration (ppm)	zone of inhibition (mm) rep 1	rep 2	rep 3
0	12	6[2]	6
0.02	14	6	6
0.04	16	11	11
0.06	17	13	14
0.08	20	16	16
0.1	22	19	17
0.2	24	21	21
0.3	26	23	23
0.4	27	26	23
0.5	30	31	25
0.6	33	27	28
0.7	32	25	30
0.8	33	32	26
0.9	33	32	29
1	36	34	33
2	38	38	38
3	42	39	41
4	45	39	42
5	47	42	45
10	50	46	46
15	55	46	49
20	56	47	49
30	60	50	51
40	62	50	52

[1] Zones of inhibition were based on the microbiological assay described in Materials and Methods.
[2] Absence of a zone of inhibition was recorded as a "6", the disk diameter. The measurement of "12 mm" in "rep 1" at 0 ppm (no tylosin) represents a false positive.

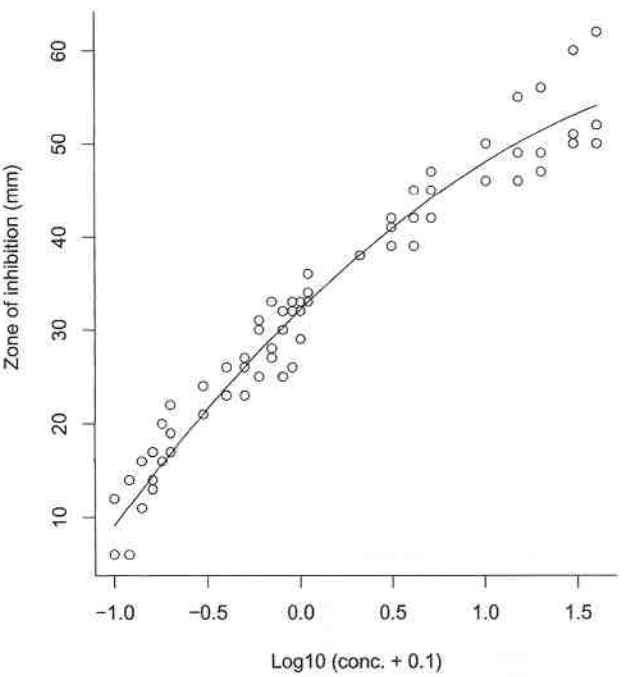

Figure 1. Tylosin standard curve. Zones of inhibition (mm) resulting from a microbiological assay are plotted against known amounts of tylosin (see Material and Methods for details); the fitted curve is overlaid.

over time in both brood and surplus honey for both the targeted (1xT) treatment dose and the 5xT dose. In surplus honey from colonies treated with the target dose, estimated tylosin concentrations declined from an average of 1.31 ppm (sampled during the treatment period) to 0.16 ppm three weeks after the last treatment. In surplus honey from colonies treated with five times the targeted dose (3000 mg total), estimated concentrations declined from 8.73 ppm to about 1.61 ppm.

DISCUSSION

Tylosin has been shown to be a safe and effective antibiotic when administered to honey bees to control the causative agent of AFB (Hitchcock *et al.*, 1970; Moffett *et al.*, 1970; Peng *et al.*, 1996; Allippi *et al.*, 1999; Elzen *et al.*, 2002). As part of a project seeking FDA approval for this antibiotic, residues in honey were determined after the application of tylosin to honey bee colonies in a sugar dust. Treatments were applied during the nectar flow, when foraging bees were returning with nectar and honey was sampled without a withdrawal period, affording FDA personnel the ability to evaluate the residue data under a worst case scenario. While several studies have previously utilized microbiological assays to examine tylosin residues and stability in honey (Máchová *et al.*, 1992; Toporcák *et al.*, 1995), differences in protocols and methodologies (*e.g.* method of application; assay conditions; limits of detection) make comparisons with our current study difficult. In our study, the estimated amount of tylosin found in honey three weeks after the last treatment with the targeted dose (200 mg in 20 g confectioner's sugar, applied three times, one week apart) was 0.16 ppm. This concentration is similar to the residue tolerances for tylosin (0.2 ppm) established by the FDA for other agricultural commodities such as chickens, turkeys, cattle, swine and eggs as reported in 21CFR556.740 of the Code of Federal Regulation (U. S. Government Printing Office, 2001). Based on a comparison of the US per capita consumption of these products in 2000 (Table III) and a tolerance of 0.2 ppm, the exposure to tylosin from honey is lower by anywhere from 12.5 (turkey) to 58.6 (beef) times than these products. In addition, it is not unreasonable to assume that tylosin exposure from honey could furthermore be reduced by establishing a usage protocol that incorporated a withdrawal period. Interestingly, we observed low levels of tylosin in honey from several untreated colonies. Since the colonies used in our study were stacked and arranged on wooden pallets in a fashion typical of colonies in US commercial apiaries, drifting bees from a treated colony to an adjacent untreated colony could account for these observations. Nonenetheless, these results indicate tylosin would prove a useful tool in combating AFB of honey bees in areas where Terramycin® is failing.

Table II. Estimates of mean tylosin concentration (ppm) in honey from colonies treated with tylosin[1]

Collection Date		03/09/01	03/16/01	03/23/01	03/30/01
		Mean Concentration in ppm (Lower,Upper 95% Confidence limits)			
Treatment[2]	Honey[3]				
1x	B		**1.45** (0.66, 3.46)	**0.47** (0.21, 1.04)	**0.40** (0.17, 0.88)
5x	B		**5.55** (2.20, 17.46)	**4.52** (1.85, 13.39)	**1.98** (0.87, 4.90)
Untreated	B		**0.12** (0.03, 0.31)	**0.00** (0.00, 0.06)	**0.00** (0.00, 0.03)
1x	S	**1.31** (0.59, 3.06)	**0.39** (0.17, 0.85)	**0.33** (0.14, 0.73)	**0.16** (0.05, 0.38)
5x	S	**8.73** (3.21, 34.27)	**3.57** (1.50, 9.90)	**2.46** (1.07, 6.33)	**1.61** (0.72, 3.85)
Untreated	S	**0.05** (0.00, 0.16)	**0.00** (0.00, 0.06)	**0.00** (0.00, 0.07)	**0.05** (0.00, 0.17)

[1] Tylosin was applied as a dust in 20 g confectioners sugar, three times, one week apart. Treatment dates were 02/23/01; 03/02/01; and 03/09/01. Estimates are based on a comparison of zones of inhibition with those obtained from the standard curve. Each mean is based upon the average of four determinations (one/colony).

[2] "1x" = 200 mg tylosin; "5x" = 1000 mg tylosin

[3] B = Brood honey; S = Surplus honey; No brood honey was collected on 03/09/01 (third treatment date).

Table III. U.S. per capita consumption (in pounds) of selected agricultural products from 1996-2000. [1]

Year	Chicken	Turkey	Beef	Pork	Eggs	Honey
1996	48.8	14.3	64.1	45.2	29.9	1.0
1997	49.5	13.6	62.7	44.8	30.2	0.9
1998	49.8	13.9	63.6	48.2	30.8	0.9
1999	52.9	13.8	64.4	49.4	32.1	1.1
2000	53.2	13.7	64.5	47.7	32.2	1.1

[1] compiled from the U.S. Economic Service (www.ers.usda.gov/foodconsumption/datasystem.asp)

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